Vol. 57, No. 12

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1991, p. 3541–3546
0099-2240/91/123541-06$02.00/0

1,3-Propanediol Production by *Escherichia coli* Expressing Genes from the *Klebsiella pneumoniae dha* Regulon

I-TEH TONG,[1] HANS H. LIAO,[2] AND DOUGLAS C. CAMERON[1]*

Department of Chemical Engineering, 1415 Johnson Drive, University of Wisconsin, Madison, Wisconsin 53706-1691,[1] and UW Biotechnology Center, University of Wisconsin, Madison, Wisconsin 53705-4098[2]

Received 11 June 1991/Accepted 30 September 1991

The *dha* regulon in *Klebsiella pneumoniae* enables the organism to grow anaerobically on glycerol and produce 1,3-propanediol (1,3-PD). *Escherichia coli*, which does not have a *dha* system, is unable to grow anaerobically on glycerol without an exogenous electron acceptor and does not produce 1,3-PD. A genomic library of *K. pneumoniae* ATCC 25955 constructed in *E. coli* AG1 was enriched for the ability to grow anaerobically on glycerol and dihydroxyacetone and was screened for the production of 1,3-PD. The cosmid pTC1 (42.5 kb total with an 18.2-kb major insert) was isolated from a 1,3-PD-producing strain of *E. coli* and found to possess enzymatic activities associated with four genes of the *dha* regulon: glycerol dehydratase (*dhaB*), 1,3-PD oxidoreductase (*dhaT*), glycerol dehydrogenase (*dhaD*), and dihydroxyacetone kinase (*dhaK*). All four activities were inducible by the presence of glycerol. When *E. coli* AG1/pTC1 was grown on complex medium plus glycerol, the yield of 1,3-PD from glycerol was 0.46 mol/mol. The major fermentation by-products were formate, acetate, and D-lactate. 1,3-PD is an intermediate in organic synthesis and polymer production. The 1,3-PD fermentation provides a useful model system for studying the interaction of a biochemical pathway in a foreign host and for developing strategies for metabolic pathway engineering.

Metabolic pathway engineering (MPE, also metabolic engineering), the modification, design, and construction of biochemical pathways, is an emerging discipline of potential importance to the chemical, biochemical, food, and environmental industries. MacQuitty (19) has called MPE the fourth wave of biotechnology following classical fermentation, recombinant DNA technology, and protein engineering. Recent progress in MPE has been reviewed by Bailey (2).

We have selected the conversion of glycerol to 1,3-propanediol (1,3-PD) as a model system for the study of MPE. Our reasons are as follows. (i) The pathway is relatively simple, consisting of only two enzymes, a dehydratase (glycerol dehydratase [EC 4.2.1.30] or diol dehydratase [EC 4.2.1.28] and 1,3-PD oxidoreductase [EC 1.1.1.202]); (ii) the pathway possesses features of a more complex metabolic network (e.g., the dehydratase is a multicomponent enzyme and requires coenzyme B_{12}, and the 1,3-PD oxidoreductase requires NADH which must be regenerated by the host cell); (iii) a large body of fundamental information is available on the 1,3-PD pathway in *Klebsiella pneumoniae* (9, 24, 26); and (iv) 1,3-PD (also known as trimethylene glycol), is a useful chemical intermediate, e.g., in the synthesis of polyurethanes and polyesters (10, 21, 32). 1,3-PD is currently derived from acrolein, a petroleum derivative, and is expensive to produce relative to other diols (6, 21).

The 1,3-PD pathway has been studied primarily in *K. pneumoniae*. Glycerol is transported into the cell through the glycerol facilitor (16). The glycerol then is converted into 3-hydroxypropionaldehyde by a coenzyme B_{12}-dependent dehydratase (22, 25, 28, 30). The 3-hydroxypropionaldehyde is reduced to 1,3-PD by an NADH-dependent 1,3-PD oxidoreductase (14). 1,3-PD is then excreted into the medium (8, 14).

The 1,3-PD pathway in *K. pneumoniae* is part of the *dha* regulon. The *dha* regulon is induced by dihydroxyacetone (DHA) in the absence of an exogenous electron acceptor, such as oxygen, fumarate, or nitrate (8). The enzymes of the *dha* regulon that are not directly involved in 1,3-PD production convert glycerol to DHA by an NAD^+-dependent glycerol dehydrogenase (13, 17) and then to dihydroxyacetone phosphate by an ATP-dependent DHA kinase (12); the dihydroxyacetone phosphate is further metabolized to provide carbon and energy for growth. The physiological reason for 1,3-PD formation is most likely to regenerate NAD^+ needed by the DHA branch of the *dha* regulon (9). *Escherichia coli* does not have a *dha* regulon; consequently, *E. coli* cannot grow anaerobically on glycerol or DHA without an exogenous electron acceptor such as nitrate or fumarate. Sprenger et al. (26) have cloned genes of the *dha* regulon in *E. coli*, but they did not detect dehydratase activity, and 1,3-PD was not produced.

In this report, we describe the construction of a cosmid containing genes from the *K. pneumoniae* ATCC 25955 *dha* regulon and the expression of these genes in *E. coli*. The production of 1,3-PD from glycerol and DHA by the transformed *E. coli* is then reported.

MATERIALS AND METHODS

Bacteria, cosmid, and enzymes. *K. pneumoniae* ATCC 25955 was used as the source of the genomic DNA. *E. coli* AG1 (F⁻ *endA1 hsdR17* [k_{12}^-, m_{k-12}^+] *supE44 thi-1 recA1 gyrA96 relA1* λ^-) (Stratagene, La Jolla, Calif.) was used as the host strain for the genomic library. Cosmid pBTI-1 (20), obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.), was used as the vector for the genomic library. Restriction enzymes were from various sources. The in vitro packaging system was obtained from Promega (Madison, Wis.), and calf intestinal alkaline phosphatase was obtained from Stratagene.

Media and growth conditions. Anaerobic fermentations were done both in Hungate tubes (18) and in anaerobic flasks

* Corresponding author.

(5) with 10 and 300 ml of liquid volume, respectively. Unless otherwise specified, growth experiments were done at 37°C with ST medium (Na_2HPO_4, 6 g/liter; KH_2PO_4, 3 g/liter; NH_4Cl, 1 g/liter; NaCl, 0.5 g/liter; $MgSO_4 \cdot 7H_2O$, 1 mM; thiamine, 0.5 mg/liter; coenzyme B_{12}, 0.5 mg/liter; $FeSO_4 \cdot 7H_2O$, 0.278 mg/liter; $ZnCl_2$, 0.136 mg/liter; $CaCl_2 \cdot 2H_2O$, 1.47 mg/liter; cysteine-HCl · H_2O, 0.5 g/liter) or modified ST medium (the same as ST medium but with 2 g of NH_4Cl per liter and 2 mM $MgSO_4 \cdot 7H_2O$) plus the appropriate carbon source(s). All fermentations also contained 50 μg of ampicillin per ml to maintain the presence of the cosmid. Anaerobic growth on agar plates was done in sealed jars under an H_2-CO_2 atmosphere (GasPak Anaerobic System; Becton Dickinson, Cockeysville, Md.).

Construction of genomic library. *K. pneumoniae* genomic DNA (10 μg) was partially digested with the restriction enzyme *Sau*3A for incubation times ranging from 15 to 60 min. After incubation at 75°C for 15 min to inactivate the enzyme, 1 μg of digested DNA was ligated with 400 ng of cosmid pBTI-1 which had been linearized by *Bam*HI and dephosphorylated by calf intestinal alkaline phosphatase. Ligated DNA (5 μl) (25% of the total ligated DNA) was mixed with 15 μl of freshly thawed packaging mix and incubated at room temperature for 2 h. The packaged DNA was diluted with 150 μl of pH 7.4 bacteriophage buffer (NaCl, 100 mM; $MgSO_4 \cdot 7H_2O$, 10 mM; Tris buffer, 20 mM) and sterilized by adding 25 μl of chloroform. Packaged DNA (10 μl) was mixed with 200 μl of exponential-phase *E. coli* AG1 grown in LB medium containing 2 g of maltose per liter and 10 mM $MgSO_4$. After incubation at room temperature for 30 min to allow the phage to absorb and to inject its DNA, 0.7 ml of SOC medium (tryptone, 20 g/liter; yeast extract, 5 g/liter; NaCl, 10 mM; KCl, 5 mM; $MgCl_2$, 20 mM; glucose, 20 mM) was added and the mixture was incubated at 37°C for 30 min. The infected *E. coli* were identified by growth on LB plates containing 50 μg of ampicillin per ml. Cells were further tested for tetracycline resistance to check the background due to vectors with no inserts. None of the 198 randomly selected ampicillin-resistant colonies that were tested still had tetracycline resistance. A primary genomic library with about 9,000 independent colonies was obtained.

Isolation of cosmid pTC1. The genomic library was enriched for glycerol- or DHA-utilizing clones by anaerobically incubating 4 ml of the primary library in 300 ml of ST medium plus 2 g of glycerol per liter and 2 g of DHA per liter. After the culture showed significant growth, cells from the enriched culture were diluted, mixed with ST medium plus 2 g of glycerol per liter, 2 g of DHA per liter, 0.1% yeast extract, and 7 g of agar per liter, overlaid on plates containing ST medium plus 2 g of glycerol per liter, 2 g of DHA per liter, 0.1% 2,3,5-triphenyltetrazolium chloride (PTPZ), and 15 g of agar per liter, and incubated at 37°C in an anaerobic jar (in retrospect, the PTPZ indicator was not necessary, but it made detection of single colonies somewhat easier). Single colonies were picked and examined for the ability to grow on glycerol plus DHA and to produce 1,3-PD. The clone which produced the highest concentration of 1,3-PD was chosen as the source of recombinant cosmid pTC1. Cosmid pTC1 was extracted and purified by following the protocol of Ausubel et al. (1) and transformed back to competent *E. coli* AG1 for long-term storage. The transformed *E. coli* AG1/pTC1 was resistant to ampicillin and was able to grow and produce 1,3-PD under anaerobic conditions on ST medium containing 2 g of glycerol per liter and 2 g of DHA per liter.

Southern hybridization. The protocol recommended by Amersham (Arlington Heights, Ill.) for Hybond-N membranes was used. ^{32}P-labeled probes were synthesized from the 2.8-kb *Hin*dIII-*Pml*I fragment of pTC1, using random primers and the Klenow fragment of *E. coli* DNA polymerase. Control probes were synthesized from the 2.1-kb *Eco*RI-*Eco*RI fragment containing the *rrsA* gene of *E. coli*. The phage clones containing the *rrsA* gene were obtained from the laboratory of Fred R. Blattner, Department of Genetics, University of Wisconsin–Madison, and were created by Kohara et al. (15).

Preparation of cell extracts. Cells from fermentation samples were centrifuged at 6,000 × *g* for 15 min, washed twice with 20 mM Tris buffer (pH 8.0), and resuspended in the appropriate suspension buffer for the enzyme to be assayed. For dehydratase activity, the suspension buffer contained 50 mM $(NH_4)_2SO_4$, 10 mM 1,2-propanediol (1,2-PD), and 10 mM potassium phosphate (pH 8.0). The 1,2-PD was added to stabilize the dehydratase (30). For 1,3-PD oxidoreductase, glycerol dehydrogenase, and DHA kinase, the suspension buffer contained 50 mM $(NH_4)_2SO_4$ and 10 mM potassium phosphate (pH 8.0). The suspended cells were disrupted by sonication (Heat Systems-Ultrasonics, Farmingdale, N.Y.), and the cell debris was removed by centrifugation. The total protein concentration was estimated by the Coomassie brilliant blue G-250 dye binding method (Bio-Rad Laboratories, Richmond, Calif.). The A_{595} was compared with that of bovine serum albumin standards (Sigma Chemical Co., St. Louis, Mo.).

Enzyme assays. The glycerol/diol dehydratase activity was estimated by the 3-methyl-2-benzothiazolinone method (31). Samples were taken 0, 2.0, 5.0, and 10.0 min. The amount of enzyme product (propionaldehyde) was determined by comparing the A_{305} to that of known standards of propionaldehyde (Sigma). One unit of activity was defined as the formation of 1 μmol of propionaldehyde per min. Diol dehydratase (EC 4.2.1.28) and glycerol dehydratase (EC 4.2.1.30) were differentiated by the method of Forage and Foster (7).

1,3-PD oxidoreductase activity was determined by the method of Johnson and Lin (14). Glycerol dehydrogenase activity was determined by the method of Ruch et al. (24). DHA kinase activity was determined by the method of Johnson et al. (12). All assays were done at 37°C.

HPLC analysis. All fermentation samples were centrifuged and filtered through a 0.45-μm-pore-size filter before analysis. 1,3-PD, ethanol, and organic acids were analyzed by high-performance liquid chromatography (HPLC) (Bio-Rad Laboratories) with an organic acids column (Bio-Rad HPX87H), using the following conditions: sample size, 20 μl; mobile phase, 0.01 N H_2SO_4; flow rate, 0.5 ml/min; column temperature, 40°C; detector, refractive index at room temperature. Sugars and glycerol were analyzed with a model 600 HPLC (Waters, Milford, Mass.) with a cation-exchange column in the calcium form (Waters Sugar-Pak II) under the following conditions: sample size, 10 μl; mobile phase, deionized water; flow rate, 0.5 ml/min; column temperature, 90°C; detector, refractive index at 35°C.

Determination of D- and L-lactate. The concentration of L-lactate in fermentation samples was measured with an enzymatic L-lactate analyzer (Yellow Springs Instrument Co., Yellow Springs, Ohio). D-Lactate concentration was estimated from the difference between the total lactate concentration measured by HPLC and the L-lactate value.

Determination of 1,3-PD by GC-MS. Samples were prepared for gas chromatographic-mass spectrometry (GC-MS) analysis by the method of Sprenger et al. (26). *E. coli* AG1/pTC1 was incubated anaerobically at 37°C in 300 ml of

ST medium plus 2 g of glycerol per liter and 2 g of DHA per liter for 140 h. Cells were removed from the medium by centrifugation, and 200 ml of the supernatant was concentrated by vacuum evaporation. The concentrate was dissolved in 30 ml of methanol, and anhydrous sodium sulfate was added to remove residual water. The sample was filtered through Whatman no. 1 filter paper and again dried by vacuum evaporation. The residual oil was redissolved in 2 ml of methanol and centrifuged in a Brinkmann microcentrifuge for 10 min to remove insoluble material. The supernatant fraction was analyzed for 1,3-PD by GC-MS on a KRATOS/MS25 instrument (Kratos Analytical Inc., Ramsey, N.J.).

GC was done on a 30-m fused silica capillary column (0.32-mm inner diameter) with 0.25-μm film thickness (SPB-5; Supelco, Inc., Bellefonte, Pa.). The injection temperature was 220°C, and the sample injection volume was 2 μl. The temperature was maintained at 50°C for 1 min and then increased by 20°C/min to 330°C. The eluted compounds were fragmented by electron impact ionization at 36 eV. The mass spectrum was compared with that of a 1,3-PD standard (Aldrich Chemical Co., Inc., Milwaukee, Wis.) obtained under the same conditions.

RESULTS

Confirmation of the presence of 1,3-PD by GC-MS analysis. *E. coli* AG1/pTC1 was grown anaerobically on ST medium plus 2 g of glycerol per liter and 2 g of DHA per liter for 140 h. Significant growth was observed (a final optical density at 660 nm of 0.162 absorbance units) and HPLC analysis of the broth showed a peak that coeluted with 1,3-PD. The final fermentation broth was analyzed by GC-MS to confirm the production of 1,3-PD. The mass spectrum of the fermentation sample gave *m/z* (relative intensity) as 59 (6), 58 (100), 57 (98), 56 (9), 55 (6), 47 (5), 46 (19), 45 (21), 44 (11), 43 (16), 31 (49), 30 (18), 29 (32), 28 (80), 27 (13). For the 1,3-PD standard, the mass spectrum was 59 (7), 58 (100), 57 (95), 56 (10), 55 (6), 47 (5), 46 (18), 45 (22), 44 (10), 43 (17), 31 (55), 30 (20), 29 (36), 28 (91), 27 (17). The results confirm that the transformed strain, *E. coli* AG1/pTC1, produces 1,3-PD. No 1,3-PD was detected in control fermentations with *E. coli* AG1/pBTI-1, i.e., cells containing the cosmid with no inserts.

In vitro activities of the *dha* regulon enzymes in *E. coli*. In *K. pneumoniae*, the *dha* regulon gene products are induced by DHA. The in vitro activities of four *dha* regulon enzymes in *E. coli* were determined in cells grown on modified ST medium plus 10 g of casein amino acids or yeast extract per liter and 2 g of a carbon source (either glycerol or xylose) per liter. Both *E. coli* AG1/pTC1 and *E. coli* AG1/pBTI-1 (cosmid vector with no inserts) were grown anaerobically in 300-ml anaerobic flasks. The activities of the four enzymes in the cell extract of *E. coli* AG1/pTC1 grown on glycerol and yeast extract were over 10-fold higher than those of *E. coli* AG1/pTC1 grown on xylose and yeast extract (noninducing conditions) and also those of *E. coli* AG1/pBTI-1 grown on yeast extract in the presence of glycerol (Table 1). The background activity of glycerol dehydrogenase in *E. coli* AG1/pBTI-1 is most likely from a dehydrogenase of unknown physiological function which converts glycerol to DHA (27); the background activity of DHA kinase is most likely from the enzyme II of the phosphoenolpyruvate-dependent phosphotransferase specific to DHA (*ptsD*) reported by Sprenger et al. (26).

The ratio of the specific dehydratase activity at 0.12 μM coenzyme B_{12} versus 12 μM coenzyme B_{12} was 0.78. By the method of Forage and Foster (7), this value indicates that 89% of the activity is from glycerol dehydratase and 11% is from diol dehydratase.

TABLE 1. Specific activities of *dha* regulon enzymes[a]

Enzyme	*E. coli* AG1/pTC1		*E. coli* AG1/pBTI-1 (glycerol + YE[c])
	Glycerol + CAA[b]	Xylose + CAA	
Glycerol/diol dehydratase	0.0016	ND[d]	ND
1,3-PD oxidoreductase	0.605	0.079	ND
Glycerol dehydrogenase	11.23	0.465	0.424
DHA kinase	13.47	0.434	0.334

[a] Specific activities in units per milligram of protein.
[b] CAA, casein amino acids, acid hydrolysate.
[c] YE, yeast extract.
[d] ND, not detectable.

Anaerobic growth and 1,3-PD production on media containing both glycerol and DHA. The time course of cell growth and 1,3-PD production by *E. coli* AG1/pTC1 grown in 300-ml anaerobic flasks on a defined medium (modified ST medium containing 2 g of glycerol per liter and 2 g of DHA per liter) is shown in Fig. 1. The specific growth rate was 0.084 h^{-1}. Growth stopped when the DHA was depleted. The yield of 1,3-PD based on the amount of both glycerol and DHA used was 0.37 mol/mol. Lactate and acetate were the dominant by-products, and no ethanol or formate was formed.

When *E. coli* AG1/pTC1 was grown on a complex medium

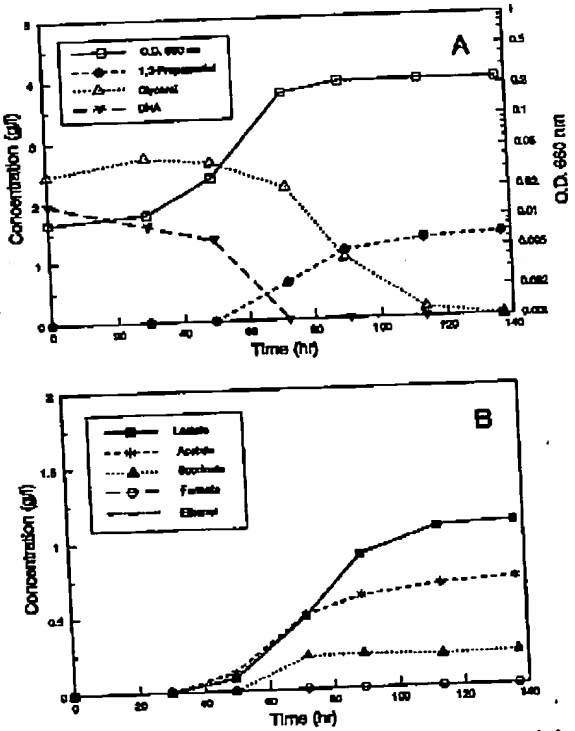

FIG. 1. Time course of *E. coli* AG1/pTC1 fermentation of glycerol and DHA on defined medium. O.D., optical density.

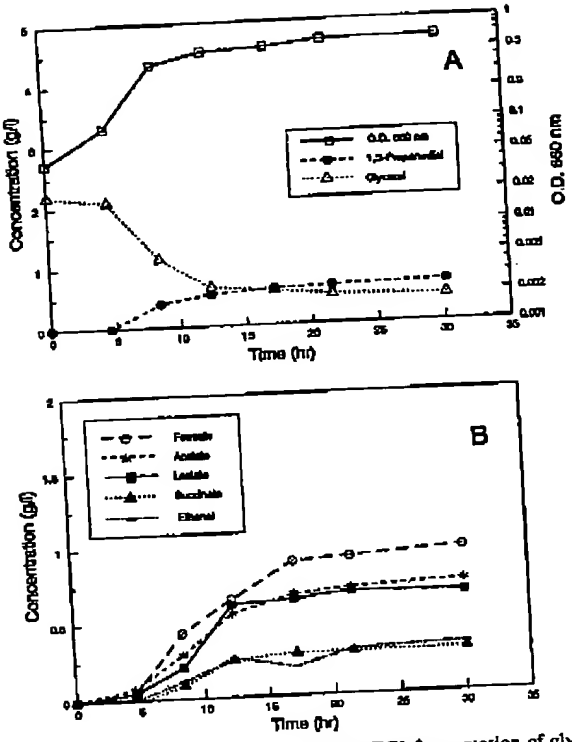

FIG. 2. Time course of *E. coli* AG1/pTC1 fermentation of glycerol on complex medium. O.D., optical density.

(modified ST medium with 2 g of glycerol per liter and 10 g of yeast extract per liter), 1,3-PD was produced from glycerol alone (Fig. 2). The growth rate was higher than that on the defined medium (0.26 h^{-1}). The yield of 1,3-PD from glycerol was 0.46 mol/mol. Lactate production was lower on the complex medium, with formate now the dominant byproduct and some ethanol accumulation. D-Lactate was the dominant form of lactate in both fermentations (0.04 g of L-lactate per liter from 1.10 g of total lactate per liter on the defined medium and 0.06 g of L-lactate per liter from 0.67 g of total lactate per liter on the complex medium).

To test whether 1,3-PD could be produced from DHA without glycerol, we grew *E. coli* AG1/pTC1 on complex medium with only DHA (modified ST medium with 2 g of DHA per liter and 10 g of yeast extract per liter) in Hungate tubes. 1,3-PD was detected with a final concentration of 0.50 g/liter; the yield of 1,3-PD from DHA was 0.31 mol/mol.

Restriction map of cosmid pTC1. A partial restriction map of the cosmid pTC1 is shown in Fig. 3. The size of the cosmid is approximately 42.5 kb. There are two copies of the vector pBTI-1 and two inserts (18.2 and 2.1 kb). This composition is probably a result of the high ratio of vector DNA to inserted DNA used in the construction of the cosmid and the large size requirement of the in vitro packaging system (at least 38 kb). We expect that the genes of the *dha* regulon are located on the major (18.2-kb) insert.

Southern hybridization. Both *K. pneumoniae* and *E. coli* genomic DNAs were digested with *Hin*dIII and *Pml*I and hybridized with the probe synthesized from the 2.8-kb

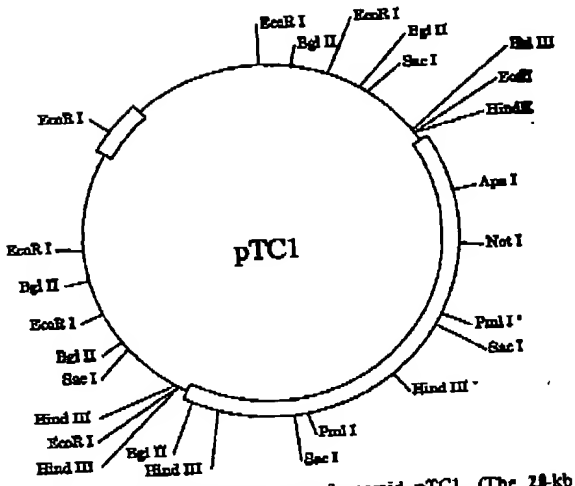

FIG. 3. Partial restriction map of cosmid pTC1. (The 2.8-kb *Hin*dIII-*Pml*I fragment used in the Southern analysis shown in Fig. 4 is indicated by asterisks.)

*Hin*dIII-*Pml*I fragment of pTC1 (Fig. 4). There are identical bands in the lane containing *K. pneumoniae* genomic DNA and the lanes containing pTC1 cosmid DNA, but no band in the lane containing *E. coli* genomic DNA. The positive control probes gave bands with both *K. pneumoniae* and *E. coli* genomic DNA. These results confirm that the insert DNA in pTC1 is from *K. pneumoniae*.

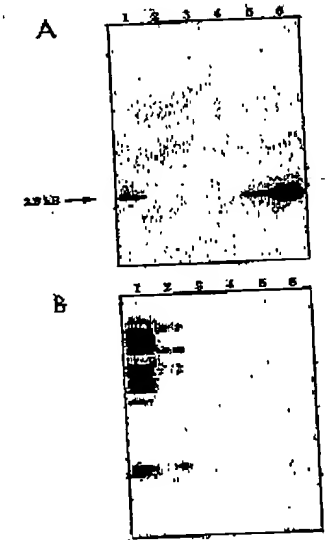

FIG. 4. Southern hybridization of *E. coli* and *K. pneumoniae* genomic DNA with probes synthesized from 2.8-kb *Hin*dIII-*Pml*I fragment of pTC1 (A) and from 2.1-kb *Eco*RI-*Eco*RI fragment containing *rrsA* (16S rRNA gene) of *E. coli* (B). All the DNA samples were digested with *Hin*dIII and *Pml*I. Lanes: 1, *K. pneumoniae* genomic DNA; 2, *E. coli* genomic DNA; 3, 10 pg of pTC1; 4, 100 pg of pTC1; 5, 1 ng of pTC1; 6, 10 ng of pTC1.

DISCUSSION

The newly constructed cosmid, pTC1, contains genes encoding for at least four enzymes from *K. pneumoniae* ATCC 25955: 1,3-PD oxidoreductase, glycerol dehydrogenase, DHA kinase, and glycerol/diol dehydratase. The first three enzymes are undoubtedly from the *dha* regulon. The situation is less clear for the dehydratase activity since both glycerol dehydratase and diol dehydratase are present in *K. pneumoniae* (7) and both enzymes catalyze the conversion of glycerol to 3-hydroxypropionaldehyde. The results of the assay for the differentiation between the two activities indicates that 89% of the activity is from glycerol dehydratase and 11% is from diol dehydratase. However, since it is unlikely that we cloned both dehydratase genes, we strongly suspect that all the activity is due to glycerol dehydratase.

E. coli AG1/pTC1 produces 1,3-PD on defined medium with glycerol and DHA, complex medium with glycerol alone, and complex medium with DHA alone. The lower yield of 1,3-PD on glycerol and DHA in defined medium than on glycerol in complex medium is expected because the yeast extract can provide carbon for cell growth and also because DHA is more oxidized than glycerol. *K. pneumoniae* ATCC 25955, the source of DNA for this study, was reported to give the same yield of 1,3-PD from glycerol in complex medium (0.46 mol/mol) (3) as did the transformed *E. coli*. This result is somewhat surprising given the differences in energy metabolism between the two organisms.

DHA is necessary for the growth of *E. coli* AG1/pTC1 on defined medium (modified ST medium with no yeast extract); the defined medium with glycerol alone did not support growth (data not shown). With both glycerol and DHA present (Fig. 1), the DHA was consumed first and then the cell level remained relatively constant while the glycerol was converted to 1,3-PD and by-products. A possible explanation for the need for DHA relates to the known bacteriostic effect of glycerol-3-phosphate (4), an intermediate produced from glycerol by the *E. coli* glycerol kinase. Cells grown on DHA may be able to counter this effect by the accumulation of fructose-1,6-diphosphate, an inhibitor of glycerol kinase (33).

The inhibition of glycerol kinase by fructose-1,6-diphosphate may also help to explain the growth of the transformed *E. coli* on the complex medium with glycerol alone (Fig. 2). The majority of the 1,3-PD was produced during rapid growth when enough fructose-1,6-diphosphate should be available from the catabolism of the yeast extract to inhibit glycerol kinase. As the available carbon source from the yeast extract is consumed, the level of fructose-1,6-diphosphate presumably decreases and the cells stop growing. There is still sufficient glycerol and other nutrients for further growth. The accumulation of by-products may also contribute to the reduction in growth. 1,3-PD production on the complex medium with DHA alone probably involved the glycerol dehydrogenase operating in the reverse of its usual direction, i.e., the oxidation of DHA to glycerol and then the conversion of glycerol to 1,3-PD in the usual way.

The by-product distribution was very different on complex versus defined medium. On the defined medium containing glycerol and DHA (Fig. 1), the major by-product was lactate, then acetate and succinate. No formate and ethanol were detected. On the complex medium (Fig. 2), formate was the major by-product and ethanol was also present. Part of this difference may be due to the rapid growth on the complex medium, resulting in the inability of the formate hydrogenlyase activity to convert all the formate to CO_2 and H_2. In both fermentations, D-lactate was the major form of lactate. The greater level of lactate in the defined medium with glycerol and DHA may be because DHA was converted to DHAP by DHA kinase and then to D-lactate via the methylglyoxal bypass (29).

The introduction of a new biochemical pathway to a cell raises many technical questions that can only be addressed experimentally. For our system, one such question was whether or not the dehydratase activity could be expressed and made to function properly in *E. coli*. Sprenger et al. (26) have reported the expression all the enzymes of the *dha* system in *E. coli* except for the dehydratase. Their organism was able to grow anaerobically on glycerol but did not produce 1,3-PD. Our success in cloning and expressing the dehydratase may be partly due to the large size of our DNA insert (18.2 kb). Another concern was that the dehydratase would be inactivated in *E. coli*. Glycerol is known to be an inhibitor of both diol dehydratase and glycerol dehydratase (22, 28). Honda et al. (11) have shown that in *K. pneumoniae* ATCC 25955 the inactivated glycerol dehydratase undergoes reactivation in situ in the presence of ATP and Mn^{2+} or Mg^{2+}. The production of 1,3-PD by our organism shows that the dehydratase is able to function in *E. coli*. The transport of glycerol and 1,3-PD by *E. coli* AG1/pTC1 is not a problem. *E. coli* is intrinsically permeable to glycerol and 1,3-PD (23).

The construction of the *K. pneumoniae* 1,3-PD pathway in *E. coli* is our first step in the development of a model system for MPE. The system will provide the opportunity to investigate the interaction of a metabolic pathway in a new host with a foreign biochemical background. It will also enable the development of methods to improve the yield and productivity of 1,3-PD from glycerol and to extend the substrate range of the pathway to more abundant renewable substrates such as sugars and starch.

ACKNOWLEDGMENTS

We thank David Snyder of the Department of Chemistry and Rowland Randall of the Department of Biochemistry, University of Wisconsin–Madison, for their assistance with the GC-MS analysis. We also thank Agnes Kanikula, Ching-Hai Kao, and Jeff Stephany for general technical assistance.

This work was supported by the National Science Foundation (BCS-8910077) and the Graduate School of the University of Wisconsin–Madison.

REFERENCES

1. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, and J. A. Smith. 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. Bailey, J. E. 1991. Toward a science of metabolic engineering. Science 252:1668–1675.
3. Cameron, D. C., I.-T. Tong, and M. Cockrem. 1990. Microbial production of propanediols. *In* Proceedings of the Corn Utilization Conference III, session I, St. Louis, Mo. National Corn Growers Association, St. Louis.
4. Cozzarelli, N. R., J. P. Koch, S. Hayashi, and E. C. C. Lin. 1965. Growth stasis by accumulated L-α-glycerophosphate in *Escherichia coli*. J. Bacteriol. 90:1325–1329.
5. Daniels, L., and J. G. Zeikus. 1975. Improved culture flask for obligate anaerobes. Appl. Bacteriol. 90:1325–1329.
6. Ehm, R., J. Falbe, H.-D. Hahn, and H.-P. Gelbke. 1980. Propandiole, p. 425–432. *In* E. Bartholomé, E. Biekert, H. Hellmann, H. Ley, M. Weigert, and E. Weise (ed.), Ullmanns Encyklopädie der technischen Chemie, vol. 19. Verlag Chemie, Weinheim.

7. Forage, R. G., and M. A. Foster. 1979. Resolution of the coenzyme B_{12}-dependent dehydratases of *Klebsiella* sp. and *Citrobacter freundii*. Biochim. Biophys. Acta 569:249–258.
8. Forage, R. G., and M. A. Foster. 1982. Glycerol fermentation in *Klebsiella pneumoniae*: functions of the coenzyme B_{12}-dependent glycerol and diol dehydratases. J. Bacteriol. 149:413–419.
9. Forage, R. G., and E. C. C. Lin. 1982. *dha* system mediating aerobic dissimilation of glycerol in *Klebsiella pneumoniae* NCIB 418. J. Bacteriol. 151:591–599.
10. Greene, R. N. June 1990. U.S. patent 4,937,314.
11. Honda, S., T. Toraya, and S. Fukui. 1980. In situ reactivation of glycerol-inactivated coenzyme B_{12}-dependent enzymes, glycerol dehydratase, and diol dehydratase. J. Bacteriol. 143:1458–1465.
12. Johnson, E. A., S. K. Burke, R. G. Forage, and E. C. C. Lin. 1984. Purification and properties of dihydroxyacetone kinase from *Klebsiella pneumoniae*. J. Bacteriol. 160:55–60.
13. Johnson, E. A., R. L. Levine, and E. C. C. Lin. 1985. Inactivation of glycerol dehydrogenase of *Klebsiella pneumoniae* and the role of divalent cations. J. Bacteriol. 164:479–483.
14. Johnson, E. A., and E. C. C. Lin. 1987. *Klebsiella pneumoniae* 1,3-propanediol:NAD^+ oxidoreductase. J. Bacteriol. 169:2050–2054.
15. Kohara, Y., K. Akiyama, and K. Isono. 1987. The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell 50:495–508.
16. Lin, E. C. C. 1976. Glycerol dissimilation and its regulation in bacteria. Annu. Rev. Microbiol. 30:535–578.
17. Lin, E. C. C., and B. Magasanik. 1960. The activation of glycerol dehydrogenase from *Aerobacter aerogenes* by monovalent cations. J. Biol. Chem. 235:1820–1823.
18. Ljungdahl, L., and J. Wiegel. 1986. Working with anaerobic bacteria, p. 84–96. *In* A. L. Demain and N. A. Solomon (ed.), Manual of industrial microbiology and biotechnology. American Society for Microbiology, Washington, D.C.
19. MacQuitty, J. J. 1988. Impact of biotechnology on the chemical industry. ACS Symp. Ser. 362:11–29.
20. Morris, D. W., J. D. Noti, F. A. Osborne, and A. A. Szalay. 1981. Plasmid vectors capable of transferring large DNA fragments to yeast. DNA 1:27–36.
21. Murphy, M. A. October 1989. U.S. patent 4,873,379.
22. Pawelkiewicz, J., and B. Zagalak. 1965. Enzymic conversion of glycerol into β-hydroxypropionaldehyde in a cell-free extract from *Aerobacter aerogenes*. Acta Biochim. Pol. 12:207–218.
23. Richey, D. P., and E. C. C. Lin. 1972. Importance of facilitated diffusion for effective utilization of glycerol by *Escherichia coli*. J. Bacteriol. 112:784–790.
24. Ruch, F. E., J. Lengeler, and E. C. C. Lin. 1974. Regulation of glycerol catabolism in *Klebsiella aerogenes*. J. Bacteriol. 119:50–56.
25. Schneider, Z., and J. Pawelkiewicz. 1966. The properties of glycerol dehydratase isolated from *Aerobacter aerogenes*, and the properties of the apoenzyme subunits. Acta Biochim. Pol. 13:311–328.
26. Sprenger, G. A., B. M. Hammer, E. A. Johnson, and E. C. C. Lin. 1989. Anaerobic growth of *Escherichia coli* on glycerol by importing genes of the *dha* regulon from *Klebsiella pneumoniae*. J. Gen. Microbiol. 135:1255–1262.
27. Sridhara, S., T. T. Wu, T. M. Chused, and E. C. C. Lin. 1969. Ferrous-activated nicotinamide adenine dinucleotide-linked dehydrogenase from a mutant of *Escherichia coli* capable of growth on 1,2-propanediol. J. Bacteriol. 98:87–95.
28. Stroinski, A., J. Pawelkiewicz, and B. C. Johnson. 1974. Allosteric interactions in glycerol dehydratase: purification of enzyme and effects of positive and negative cooperativity for glycerol. Arch. Biochem. Biophys. 162:321–330.
29. Tempest, D. W., and O. M. Neijssel. 1987. Growth yield and energy distribution, p. 797–806. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
30. Toraya, T., and S. Fukui. 1982. Diol dehydratase, p. 233–262. *In* D. Dolphin (ed.), B_{12}: biochemistry and medicine. John Wiley & Sons, Inc., New York.
31. Toraya, T., K. Ushio, S. Fukui, and H. P. C. Hogenkamp. 1977. Studies on the mechanism of the adenosylcobalamin-dependent diol dehydratase reaction by the use of analogs of the coenzyme. J. Biol. Chem. 252:963–970.
32. Whinfield, J. R., and J. T. Dickson. March 1949. U.S. patent 2,465,319.
33. Zwaig, N., W. S. Kistler, and E. C. C. Lin. 1970. Glycerol kinase, the pacemaker for dissimilation of glycerol in *Escherichia coli*. J. Bacteriol. 102:753–759.